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Determination of the Plasma Protein Binding of New Psychoactive Substances

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Drug Test Anal. 2016;

Table of Contents

List of Abbreviations

APCI	Atmospheric pressure chemical ionization
CB1	Cannabinoid receptor type 1
CI	Confidence interval
CV	Coefficient of variation
D1	Dopamine receptor D1
D2	Dopamine receptor D2
D9-THC	D9-Tetrahydrocannabinol
EU	European Union
HP	Human plasma
IS	Internal standard
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LC-MSD	Liquid chromatography mass spectrometry device
LogP	Partition coefficient
MaxP	Maximal pressure
MDMA	3,4-Methylendioxy-N-methylamphetamin
MS	Mass spectrometry
M/z	Mass-to-charge ratio
NPS	Novel psychoactive substances
PDE4	Phosphodieseterase-4
P-gp	P-glycoprotein
PPB	Plasma protein binding
RT	Retention time
RSD	Relative standard deviation
SC	Synthetic cannabinoids
SD	Standard deviation
SIM	Select ion monitoring
SN	Supernatant
THC	Tetrahydrocannabinol
UF	Ultrafiltrate

List of Tables

1 Summary

Introduction Only free drugs can cause pharmacological effects and can be metabolized and excreted but the binding to plasma proteins is usually only studied for therapeutic drugs. However, for prediction of drug-drug interaction or estimation of metabolic stability this parameter is of importance. Thus, this study aims to develop an easy and quick procedure to determine the plasma protein binding of drugs of abuse, particularly of new psychoactive substances.

Methods The degree of plasma protein binding was determined using ultrafiltration and liquid chromatography-mass spectrometry (LC-MS) analysis of the unbound fraction. Millipore Centrifree two-chambered devices with a semipermeable membrane were used for ultrafiltration. An Agilent MSD was used for MS analysis after separation by reversed phase LC. Sample preparation after centrifugation consisted of dilution and protein precipitation using acetonitrile. Codeine was added as an internal standard for all analytes. Chromatographic separation was achieved using a gradient elution of ammonium formate buffer and acetonitrile. Determination of PPB was then carried out by analysis of at least six replicates at analyte concentration of 5 µM prior and after ultrafiltration. Linear calibration curves using concentration levels from 1-5 µM were prepared in plasma and ultrafiltrate and processed as described above.

Results Plasma protein binding has successfully been determined for 15 NPS representing four different classes (synthetic cathinones, synthetic cannabinoids, research chemicals and herbal drugs). Values reached from 67% for benzedrone to >99% for WIN 55,212-2. Due to the importance of the free and bound ratio of drugs in human blood, this method can serve as a fast and reliable technique for determination of their plasma protein binding in forensic and clinical toxicology.

2 Zusammenfassung

Einleitung: Nur der ungebundene Anteil eines Stoffes ist pharmakologisch wirksam und kann im Körper metabolisiert und ausgeschieden werden. In der Literatur existieren grundsätzlich vorwiegend Daten über die Plasmaproteinbindung für Arzneistoffe. Dabei ist die Plasmaproteinbindung gerade zur Beurteilung der metabolischen Stabilität eines Stoffes und dessen Wechselwirkungen mit anderen Substanzen essentiell. Die vorliegende Arbeit dient folglich der Entwicklung eines einfachen und schnellen Verfahrens zur Bestimmung der Plasmaproteinbindung von Missbrauchsdrogen, insbesondere von neuen psychoaktiven Substanzen.

Methodik: Das Ausmaß an Plasmaproteinbindung wurde mithilfe von Ultrafiltration und Flüssig-Chromatographie mit Massenspektrometrie-Kopplung der ungebundenen Fraktion ermittelt. Alle Experimente wurden unter Verwendung von Millipore Centrifree zwei-Kammer-Ultrafiltrationseinheiten mit semipermeabler Membran und einem Agilent MSD zur massenspektrometrischen Analyse nach erfolgter Stofftrennung durch Umkehrphasen-Flüssig-Chromatographie durchgeführt. Nach Zentrifugation erfolgte die Probenaufbereitung durch Proteinfällung mit Azetonitril. Als interner Standard für alle Analyten wurde Codein benutzt. Die chromatographische Auftrennung wurde durch Gradientenelution mit Ammoniumformatpuffer und Azetonitril erreicht. Die Bestimmung der Plasmaproteinbindung gelang durch Analyse von sechs gleichen Proben vor und nach Ultrafiltration mit einer Ausgangskonzentration jedes Analyten von 5 µM. Lineare Kalibrationsgeraden im Konzentrationsbereich von 1-5 µM wurden in Humanplasma und Ultrafiltrat erstellt. Das weitere Vorgehen war wie oben beschrieben.

Ergebnisse: Die Plasmaproteinbindung wurde erfolgreich für 15 neue psychoaktive Substanzen aus vier verschiedenen Drogenklassen ermittelt. Vertreten sind synthetische Cathinone, synthetische Cannabinoide, Research Chemicals und Herbal Drugs. Die ermittelten Werte erstrecken sich von 67% für Benzedrone bis >99% für WIN 55,212-2. Aufgrund der klinischen Relevanz des Plasmaprotein-bindungsverhaltens von Missbrauchsbrogen, kann die vorliegende Arbeit für diesen Zweck als einfach realisierbares und sicheres Verfahren in der forensischen und klinischen Toxikologie dienen.

3 Introduction

3.1 Plasma Protein Binding

Plasma protein binding (PPB) is a decisive factor for the efficacy and toxicology of a drug considering the fact that only the unbound drug interacts with the target receptor achieving the desired effect (1–4). High binding values to plasma proteins reduce the concentration of the free drug in the systemic circulation (5). Furthermore, drug-protein complexes serve as drug reservoir and prolong the half-life period, respectively (1,6). Volume of distribution, biotransformation, and elimination are based on free drug concentration and could result in considerable modifications depending on PPB (5,6). In addition, the balance between bound and unbound concentration is affected by changes in pH value, temperature, and the concentration of both plasma proteins and competitive drugs (4). Alteration in PPB, such as renal or hepatic impairment or pregnancy, can lead to acute toxicity (5,7,8). The two predominant binding proteins are albumin and α1-acid glycoprotein, whereby albumin represents nearly 60% of the total protein in human plasma. Its concentration in plasma is almost constant, but can reduce promptly after severe injuries and surgery (4). α1-acid glycoprotein is an acute-phase-protein generally increasing in infectional and inflammatory diseases (1,4). Drug-drug interactions based on competition for the same binding sites result in a displacement from bound molecules and an increased blood level of the free drug (1,7). This exhibits possible toxicological effects in case of any additional drug use (5).

3.2 Novel Psychoactive Substances

In recent research, the term *novel psychoactive substances* (NPS) is used for occurring synthetic drugs of abuse typically designed as chemical derivatives of well-known illicit stimulants such as cocaine, amphetamine, or cannabis (9–12). NPS create similar pharmacological effects to the drugs they imitate but with the advantage of legal status in many states and the ready availability from head shops or the online market sold as bath salts or herbal mixtures (9–13). Key reasons for the increasing popularity include above all the difficulty of detecting NPS by analysis of biological materials in many standard laboratories (11–15). This fact makes newly marketed drugs of abuse particularly attractive for persons frequently undergoing drug screenings as it is seen in withdrawal clinics (16).

More than 300 different NPS have been synthesized since the beginning of the 21th century (10–12). In the European Union (EU), researchers have been identifying more than one additional designer drug every week due to quick replacement of

recently banned drugs by a similar substance on the market (11). NPS are associated with higher risk of addiction, overdose, and adverse health effects (10). Because of the varying quantities of numerous compounds, found within the marketed product, risk of poisoning associated with hospitalization or death, is highly prevalent (12,13,17–19).

Due to the complexity of examining effects drugs of abuse have on humans, little human studies with reference to their pharmacology and toxicity exist (8,12,13,15). Against the background of the fast identification and quantification of psychoactive drugs, a diversity of methods, particularly mass spectrometry (MS), are reported in various essays as sensitive and selective (6,7,14,20–22). At present, there is no data available on PPB for designer drugs, especially NPS. Therefore, PPB is considered as an important parameter to establish safety margins for drug use and was determined for 15 NPS representing four different classes (synthetic cathinones, synthetic cannabinoids, research chemicals and herbal drugs) to acquire valuable knowledge about their distribution, delay of elimination, and acute risk of toxicity.

3.3 Spectrum of NPS

3.3.1 Synthetic Cathinones

The naturally occurring alkaloid cathinone is commonly found in shrubs, namely in *Catha edulis*. This type of plant only growing in parts of East Africa and the Arabian Peninsula, has been noticed by Europeans because of its psychostimulant effect (9,23). Cathinones derive from the phenylethylamine family, only differing in a ketone group at the β-position of the amphetamines (9,11,23). Synthetic cathinones are potent norepinephrine, serotonin, and dopamine reuptake inhibitors and they are responsible for neurotransmitter release (9–11,23,24). Desirable effects are as follows: euphoria, increased endurance as well as reduced appetite and need to sleep (9,10,13,14). Several synthesized cathinones were patented for therapeutic purposes notably as appetite suppressants (9,14). However, with increasing cases of abuse and reports of acute toxicity, legal restrictions were enforced and individual derivates were submitted to control measures across the EU in 2010 (9). Nowadays, there are approximately 30 known cathinone derivates (13), found as the main components of so-called bath salts. Compared with the non-β-keto amphetamines, synthetic cathinones possess a high affinity towards the dopaminergic receptors, thus triggering abuse and addiction (9–11).

Synthetic cathinones can be classified based on their pharmacological properties. Five compounds are investigated in this research for their PPB profile (cf. Fig.10). Since 2007, mephedrone has been the most frequently found synthetic

cathinone available on the European market (9,13). Along with benzedrone, it belongs to a class characterized by its activity spectrum similar to cocaine and MDMA (10,11). In 2008, the first death caused by the abuse of mephedrone has been confirmed in Sweden (9). Methylone is the β-keto derivative of MDMA representing a large group of analogues of 3,4,-methylenedioxyamphetamines (9). This compound is known to be the main ingredient of the liquid designer drug *Explosion* (9) and referred to as MDMA-like entactogens and empathogens, causing altered visual, auditory, and tactic perception. Furthermore, MDPV and PVP belong to the widely spread derivates of the synthetic cathinone pyrovalerone (11). MDPV is at least 10-fold more potent compared with cocaine (25). Acute toxic effects are predominantly related to the potent stimulation of the catecholaminergic system (23). Acute psychotic symptoms include agitation, epileptic seizures, hallucination, and cardiovascular toxicities (11,23,25). A widespread of MDPV has been observed as a recreational drug leading to increasing rates of acute intoxication and death, particularly among young adults (23).

3.3.2 Synthetic Cannabinoids

Synthetic cannabinoids (SC) consist of a huge and fast growing class of NPS characterized by their high affinity to the CB1 receptor (11) and marketed as legal alternatives to D9-tetrahydrocannabinol (D9-THC) (21). Herbal cannabis is extensively consumed because of its psychotropic potentials such as euphoria, intensified perception, and increasing appetite. SC have primarily been designed in research centers in order to explore the endocannabinoid pathway (22,26). Considering the spread of SC in herbal mixtures, they have rapidly developed into recreational drugs (22). Being a group of over 50 known compounds with different structures (22), SC operate as potent cannabinoid receptor agonists with mostly stronger CB1 binding affinity and higher analgesic potency than other cannabinoids (11,17,22). On the other hand, the misuse of SC commonly results in agitation, tachycardia, chest pain, and nausea and specifically acute psychosis and paranoia (22). Severe toxicity includes seizures, hallucinations, hypokalemia, and acute kidney failure (11,18,22). Seven frequently misused SC representing agents of three significant chemical families are investigated for their affinity to plasma proteins. They include the naphhoylindoles, benzylindoles and indazole-carboxamides (cf. Fig. 11).

Most SC are named JWH which derives from the initials of Dr. John William Huffmann, who synthesized numerous psychotropic cannabinoids according to the family of aminoalkylindoles in 1994 (10,22). Being an integral part of more than 60 different drug brands in Germany (18), JWH-018 was the first identified compound in

the herbal mixture named *Spice* (10). JWH-018 structurally differs from JWH-210 and AM-2201 to a lesser extent. It is one of the most potent naphthoylindole-derivates leading to strong mental and physical effects and high risk of intoxication (18,22). AM-2201 was synthesized to undergo common drug screenings after having achieved detectability of JWH-018 in urine samples (16). Research proves that the combination of alkyl substituents at C2 of the indole with bicyclic aroyl groups is responsible for an increase of cannabinoid potency (27). JWH-200, a further representative of the naphthoylindole-family is one of the first synthesized SC of its kind providing a more complex composition. With the intention to investigate numerous derivates of the aminoalkylindoles, the Winthrop group synthesized miscellaneous SC including WIN 55-212,2 (27). As shown by Meyer et al., JWH-200 and WIN 55-212,2 were both identified as potent inhibitors of p-glycoprotein (P-gp) (28). In contrast, RCS-4 and AB-PINACA-5F both vary from the reported basic structure of the naphtoylindole family. In order to compare the interaction with plasma proteins between different chemical types of SC, the described compounds are studied in the following experiments.

3.3.3 Research Chemicals

So-called research chemicals are created in common laboratories for pharmacological and therapeutic purposes, but are widely misused as recreational designer drugs with typically hallucinogenic properties. Two significant structural families of hallucinogens and their synthesized derivatives exist: indolamines and phenylalkylamines (12) (cf. Fig.12). 25I-NBOMe was developed by Ralf Heim at the University of Berlin as potent agonist for 5-HT_{2A} serotonin receptors with hallucinogenic activity in 2003 (19). 25I-NBOMe belongs to the chemical family of 2,-5-dimethoxyphenethylamines, called the *2C compounds* whereof 25I-NBOMe has been one of the most potent compounds since 2012 (11,19). Its abuse has been attributed to severe serotonergic-, but also to sympathomimetic intoxications (11) as well as death cases mostly caused by overdose due to its extremely low microgram dosage (19). 5-MeO-DALT is part of the huge class of hallucinogenic tryptamines synthesized by the chemist Alexander Shulgin in 2004 (12). Tryptamines contain an indole nucleus as basic structure combined with an ethylamine group (11). Hallucinogenic tryptamines have high structural similarities with serotonin and a high affinity for the serotonin 5-HT_{2A} receptor (12). The indole nucleus has proven to be responsible for the hallucinogenic properties (12). Slight alterations of the chemical structure lead to new substances with more powerful psychoactive effects (12). Up until today, about 50 novel synthetic tryptamines have been known for their common misuse and as a substitute for traditional hallucinogens (12). All

hallucinogenic tryptamines produce changes in visual perception, feeling of unreality, and depersonalization provoking psychotic panic reactions (12). Acute toxic effects include severe agitation, excited delirium, anterograde amnesia, and catalepsy. The consumption of 5-MeO-DALT is only outlawed in a few countries around the world (12).

3.3.4 Herbal Drugs

Herbal drugs span a wide range of recreational drugs obtained from psychoactive alkaloids. Representatives are legally marketed despite causing addiction and cognitive impairments (29–31). Glaucine is the main component of *Glaucium flavum* (yellow horn poppy) of the Papaveraceae family growing in Western Europe, North America and Asia, as well as therapeutically used as cough suppressant in Bulgaria and neighboring countries (31). It is known to exhibit a wide spectrum of pharmacological activities as it acts as a PDE_4 inhibitor and calcium channel blocker as well as central dopamine D1 and D2 receptor antagonist (31). Furthermore, Glaucine possesses a high inhibitory potential on P-gp comparable with verapamil (28). This might cause serious interactions with simultaneously administered drugs as glaucine decreases the efflux ratio of P-gp and, as a consequence, increases bioavailability of P-gp substrates (28). Likely misused as recreational drug, consumption of Glaucine is accompanied by increasing cases of toxicity including hallucinations and dissociative-type symptoms (31).

3.3.5 Aims of the Present Study

1) Establishing an easy and quick technique for determination of PPB

2) Verification of the technique with positive control compounds

3) Determination of PPB of 15 NPS

4) Comparison of binding affinity between the respective groups of NPS

4 Experimental Procedures

4.1 Chemicals and Reagents

PVP *(1-phenyl-2-pyrrolidin-1-ylpentan-1-one)* was purchased from Pierce Kavanagh, Ph.D., Trinity Centre of Health and Science (Dublin, Ireland). MDPV *(1-(1,3-benzodioxol-5-yl)-2-pyrrolidin-1-ylpentan-1-one)* was provided by LGC Standards GmbH (Wesel, Germany). Methylone *((2R)-1-(1,3-benzodioxol-5-yl)-2-(methylamino) propan-1-one)* was purchased from Israel Police HQ (Jerusalem, Israel) for research purposes. All other investigated synthetic cathinones were obtained from commercial suppliers. These include mephedrone *((2S)-2-(methylamino)-1-(4-methylphenyl) propan-1-one)*, and benzedrone *(2-(benzylamino)-1-(4-methylphenyl)-propan-1-one)*. JWH-018 *(naphthalen-1-yl-(1-pentylindol-3-yl) methanone*), JWH-210 *((4-ethyl-naphthalen-1-yl)-(1-pentylindol-3-yl)methanone*), JWH-200 *([1-(2-morpholin-4-ylethyl) indol-3-yl]-naphthalen -1-ylmethanone)*, RCS-4 *((4-Methoxyphenyl)(1-pentyl-1H-indol-3-yl)methanone)* and AM-2201*([1-(5-fluoropentylindol-3-yl]-naphthalen-1-ylmethanone)* were provided by LGC Standards GmbH (Wesel, Germany). WIN 55,212-2 mesylate (purity 99*%) ((2,3-dihydro-5-methyl-3-((4-morpholinyl)methyl)pyrrolo-(1,2,3-de)-1,4-benzoxazin-6-yl)(1-naphthalenyl) methanone)* was purchased from THC Pharm GmbH (Frankfurt am Main, Germany). AB-PINACA-5F *(N-[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-1-(5-fluoropentyl)-indazole-3-carboxamide)* was obtained from Prof. Dr. rer. nat. Dipl.-Chem. Volker Auwärter, Institut für Rechtsmedizin (Freiburg, Germany). 25I-NBOMe *(2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl-)methyl]ethanamine)* was purchased from LGC Standards GmbH (Wesel, Germany). 5-MeO-DALT *(N-[2-(5-methoxy-1H-indol-3-yl)-ethyl]-N-prop-2-enylprop-2-en-1-amine)* was provided by Dr. Simon Brandt (Liverpool John Moores University, UK). Glaucine *((6aS)-1,2,9,10-tetramethoxy-6-methyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinoline)* was obtained from Oskar Tropitzsch GmbH (Marktredwitz, Germany). Water was purified in the authors' laboratory using a Millipore filtration unit. All other chemicals and reagents were from E. Merck (Darmstadt, Germany) and they were of analytical grade.

4.2 Ultrafiltration Conditions

PPB was investigated using two-chambered *Centrifree* ultrafiltration devices purchased from *Merck Millipore* (Darmstadt, Germany). Experimental conditions were adapted from Fung et al. (32) and modified as described below. Each unit consisted of a low-adsorptive hydrophilic membrane and an O-ring between a sample reservoir on the top and a removable filtrate cup on the base. The filter consisted of *Ultracel PL*

regenerated cellulose membranes with a diameter of 14 mm and an active membrane surface of 0.92 cm^2. The human plasma samples containing the dissolved drug were transferred to the upper chamber. Accelerated through ultracentrifugation, the sample was soaked through the filter chosen to let only the unbound drug pass. Larger molecules as the protein bounded compound were retained and strict separation between the protein-free ultrafiltrate and the protein bound compound was finally achieved. The molecular weight cut-off amounted to 30 kDa. The recommended sample volume was up to 1000 µL and for the best results it was required to operate at relative centrifugal force of 1000-2000 x g[1].

4.3 Liquid Chromatography Conditions

Reversed phase liquid chromatography (LC) based on modified silica gel was applied to attain separation of the substances to be analyzed. The altered sorbent was characterized by its nonpolar nature through the use of octyl chains as functional group. The applied *Superspher® 60 RP-select B* spherical porous microparticles with a particle size of 4 µm were packed into a *Merck LiChroCART column* (125 mm × 2 mm internal diameter) and a pre-connected *LiChroCART 10–2 Superspher 60 RP Select B* guard column. The mobile phase consisted of 50 mM ammonium formate (3.15 g/L) adapted to pH 3.5 with formic acid (eluent A) and acetonitrile containing 1 mL/L formic acid (eluent B). After de-gassing, the mobile phase was moved by a pump with a maximal pressure limit of 400 bars while keeping a flow rate of 0.4 ml/min. The temperature was set at 25°C and the injection volume was 10 µL. Chromatographic separation was achieved according to the retention time of each compound in the stationary phase and the speed of desorption in the eluent mixture. During the analysis the gradient was programmed as listed in Table 1. Table 2 shows the modified gradient used for chromatographic separation of methylone and mephedrone demonstrating a very close retention time to codeine in the standard settings.

Tab. 1 Standard LC-Timetable with indication of ratio of eluent B, %, flow rate and maximal pressure (MaxP) at the relevant time, minutes.

Time	%B	Flow	MaxP
0.00	40	0.400	400
4.00	40	0.400	400
4.01	90	0.600	400
7.00	90	0.600	400
7.01	40	0.400	400
10.00	40	0.400	400

[1] Based on the recommendation of *Centrifree Ultrafiltration Devices* (User Guide).

Tab. 2 LC-Timetable applied for mephedrone and methylone

Time	%B	Flow	MaxP
0.00	1.0	0.700	400
0.50	1.0	0.700	400
3.00	30	0.700	400
4.50	30	0.700	400
5.00	40	0.700	400
5.01	99	0.700	400
6.00	99	0.700	400
6.01	1.0	0.700	400
9.00	1.0	0.700	400

4.4 LC-MS Conditions

The samples were quantified using an *Agilent Technologies* (AT, Waldbronn, Germany) *AT 1100 series* LC-MSD, SL version, defined as a quadrupole based atmospheric pressure chemical ionization (APCI) instrument and controlled by *LC-MSD ChemStation* using the A.08.03 software. The general conditions were adapted according to Maurer et al. (33). APCI is characterized by direct and continuous transfer of the liquid sample into vapor phase while maintaining atmospheric pressure (34). Therefore a unity of pneumatic nebulizer and heated spray chamber with a fine needle electrode was applied as ion source. Device setup comprised the following:

Drying gas: nitrogen (7000 mL/min, 300°C); nebulizer gas: nitrogen (25 psi, 172.3 kPa); vaporizer temperature: 400°C; capillary voltage: 4000 V; corona current: 5.0 mA; fragmentor voltage: 100 V.

A solution comprising the analyte was injected in hot nitrogen current to vaporize the solvent. Ion generation was achieved by corona discharge between the needlepoint and spray chamber in counter electrode function. The analytes were identified and quantified by the selected ion monitoring (SIM) mode using ions at the respective m/z $(M+H)^+$. *GraphPad Prism 5* (GraphPad Software Inc., San Diego, California) was used for calculating descriptive statistics and linear regression models. Quantification was performed using an unweighted linear regression model. Calibration curves were constructed plotting peak area ratios (analyte against codeine, m/z 300) of spiked calibrators both in human plasma (1, 2, 3, 4, and 5 µM) an ultrafiltrate (1, 2, 3, 4, and 5 µM) versus their concentrations. As the class of SC suggested high PPB with regard to THC, their calibration model in ultrafiltrate was selected to range from 0.2-1

µM. MS response peak area ratios (NPS against IS) versus concentration were plotted to obtain calibration curves.

4.5 Study Design

4.5.1 Calibration Standards in Human Plasma and Ultrafiltrate

All tested NPS were prepared as a 10 µM initial pure substance solution whereof a solution series in the concentration range of 1-5 µM was done both in blank human plasma and blank ultrafiltrate. At first, spiked human plasma was incubated in 1.5 ml *Eppendorf* tubes for 30 minutes at 37°C in a compact thermo shaker. Protein precipitation was achieved by diluting 100 µL of the spiked samples with 200 µL acetonitrile (2M) und 100 µL ammoniumacetate (2M). As internal standard (IS), codeine dissolved in acetonitrile was used at a final concentration of 2 µM. At 1680 rpm, the tubes were vortexed and centrifuged for 90 seconds. Thereafter, 100 µL of the clear supernatant were transferred to glass microvials and analyzed by LC-MS. Reverse phase liquid chromatographic separation was achieved using a gradient elution of ammonium formate buffer and acetonitrile. Calibration curves were constructed using MS response peak area ratios (analyte/codeine) against the target concentration. A straight-line equation (y=m*x) for calibration was calculated for all investigated compounds in human plasma using concentration levels from 1-5 µM. To generate the ultrafiltrate, 500 µL of human plasma (n=6) were added to *Centrifree*-devices and centrifuged for 30 min at 3000 rpm. The thus gained ultrafiltrate in each device was spiked with the analyte in order to obtain a concentration range of 1-5 µM. A volume of 100 µL of the spiked ultrafiltrate was treated as described above for determination of calibration curves in ultrafiltrate.

4.5.2 Sample Preparation for PPB Studies

PPB was investigated by comparison of drug concentrations prior and after ultrafiltration. Six replicates of human plasma (500 µL) spiked with the tested compound at a defined concentration of 5 µM after incubation were split into two different test series, respectively. A control series was prepared by vortexing and centrifuging 100 µl of each sample treated previously with acetonitrile for protein precipitation and spiked with the IS as described above. The six human plasma samples were analyzed via LC-MS by integration of the desired fragment ions and

applying the established human plasma calibration equation in order to verify the initial concentration of 5 µM.

To determine the PPB on the basis of the free drug concentration in ultrafiltrate, 400 µl of the incubated samples were centrifuged in *Centrifree* devices as described above. 100 µl of the thus gained ultrafiltrate in each device were prepared similarly to the control and calibration series and transferred to glass vials to be analyzed by LC-MS. The unbound concentration was directly calculated based on MS response area ratios applied to the ultrafiltration calibration equation made for each tested compound. To calculate the unbound fraction, the total concentration of the respective compound in human plasma was added to the relation and PPB could be determined according to the following equation:

$$\mathbf{PPB\ (\%) = \left(1-\left(\frac{C\ UF}{C\ HP}\right)\right)* 100}$$

C *UF* = Drug concentration in ultrafiltrate (µM)
C *HP* = Drug concentration in human plasma (µM)
$\frac{\mathbf{C\ UF}}{\mathbf{C\ HP}}$ = Ratio of unbound tested drug

To double check the results, the concentration of the retained volume in the *Centrifree* devices was determined in the same way for any tested drug. The applied method was verified as follows:

$$\mathbf{C\ UF + C\ RV = 5\ \mu M}$$

C *UF* = Drug concentration in ultrafiltrate (µM)
C *RV* = Drug concentration in retained volume (µM)

All experimental steps were verified using representatives of drugs with already existing PPB values in literature (22,35,36). PPB studies on sertraline, cimetidine, DOI, butylone, and THC were performed to assure that the applied method was sensitive and reliable for the provided study.

5 Results

5.1 Chromatographic Separation

Chromatographic separation was achieved according to the retention time of each compound in the stationary phase and the speed of desorption in the eluent mixture. The eluting power of the mobile phase rose with decreasing polarity by augmenting the concentration of the stronger eluent B during the separation process. In order to achieve strictly separated signals of drug and IS, the gradients of eluent A and B during

the separation process were programmed as already described. A new gradient was needed for methylone and mephedrone, which showed a very close retention time to codeine in the standard setting. By increasing the flow rate and rapid increase of eluent B in gradual steps, well separated and sharp signals could be obtained as shown in Figure 2. The analytes were quantified by the SIM mode using the protonated molecular ions at m/z $(M+H)^+$. These m/z were found to be selective and provided the most intense signal in the mass spectrometer. The respective target ions and retention times obtained by LC-MS are summarized in Table 4. Representative chromatograms for the two applied LC-MS settings are shown in Figures 1 and 2 exemplified for synthetic cathinones at a concentration of 2 µM in the ultrafiltrate. Retention times are given on the abscissa whereas the y-values depend on the respective ion abundance at this point.

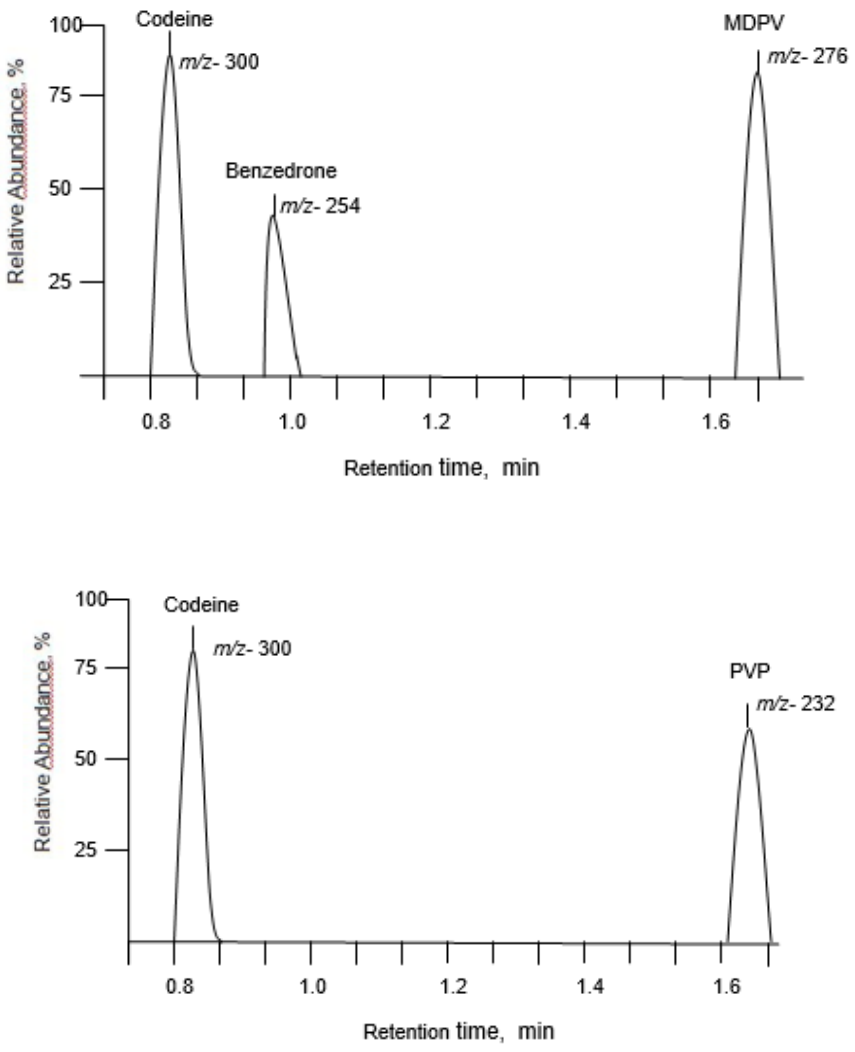


Fig. 1 Representative LC-MS-chromatograms of synthetic cathinones and internal standard (IS) obtained by standard LC-setting and quantification in SIM-mode using m/z-: *232 (PVP); 254 (benzedrone); 276 (MDPV); 300 (codeine, IS).* The chromatograms are of 2 µM of the calibrator in ultrafiltrate

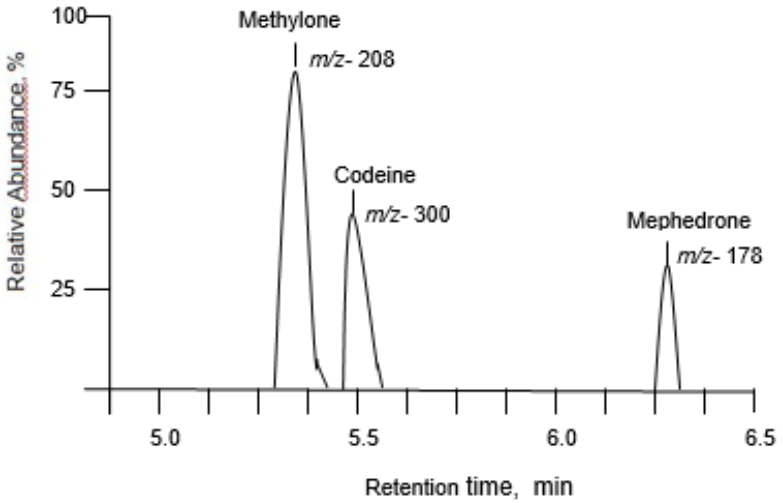


Fig. 2 Representative LC-MS-chromatogram obtained by the modified LC-setting optimized for methylone and mephedrone m/z-: *178 (mephedrone); 208 (methylone); 300 (codeine, IS)*. The chromatogram is of 2 µM of the calibrator in ultrafiltrate

Tab. 3 NPS with corresponding formula and exact masses, targeted ions in the SIM mode and retention time (RT) recorded by LC-MS

NPS	Formula	Exact mass, g/mol	SIM Ion	RT, min
Mephedrone	$C_{11}H_{15}NO$	177.12	178	6.3
Methylone	$C_{11}H_{13}NO_3$	207.09	208	5.5
Benzedrone	$C_{17}H_{19}NO$	253.15	254	0.9
MDPV	$C_{16}H_{21}NO_3$	275.15	276	1.7
PVP	$C_{15}H_{21}NO$	231.16	232	1.7
JWH-018	$C_{24}H_{23}NO$	341.18	342	7.1
JWH-210	$C_{26}H_{27}NO$	369.21	370	7.2
AM-2201	$C_{24}H_{22}FNO$	359.17	360	6.7
JWH-200	$C_{25}H_{24}N_2O_2$	384.18	385	3.5
WIN-55,212-2	$C_{27}H_{26}N_2O_3$	426.19	427	6.6
RCS-4	$C_{21}H_{23}NO_2$	321.17	322	6.6
AB-PINACA-5F	$C_{18}H_{25}FN_4O_2$	348.19	349	4.8
25I-NBOMe	$C_{18}H_{22}INO_3$	427.06	428	4.7
5-MeO-DALT	$C_{17}H_{22}N_2O$	270.17	271	1.7
Glaucine	$C_{21}H_{25}NO_4$	355.18	356	1.4

5.2 Method Verification

The experiments were first performed using five positive control compounds (n=6) with already known PPB (22,35,36). The selected values range from 15% to 97% thus covering low, medium, and high PPB. Table 3 shows experimental means ± standard deviation of all tested compounds. The 95% confidence intervals (CI) of the means were within 80-110% of the respective target values taken from literature (22,35,36). Accuracy and precision data were both within the acceptance interval of 15% for the five positive control compounds; except for cimetidine showing a relative standard deviation (RSD) exceeding the accepted limits of 15%.

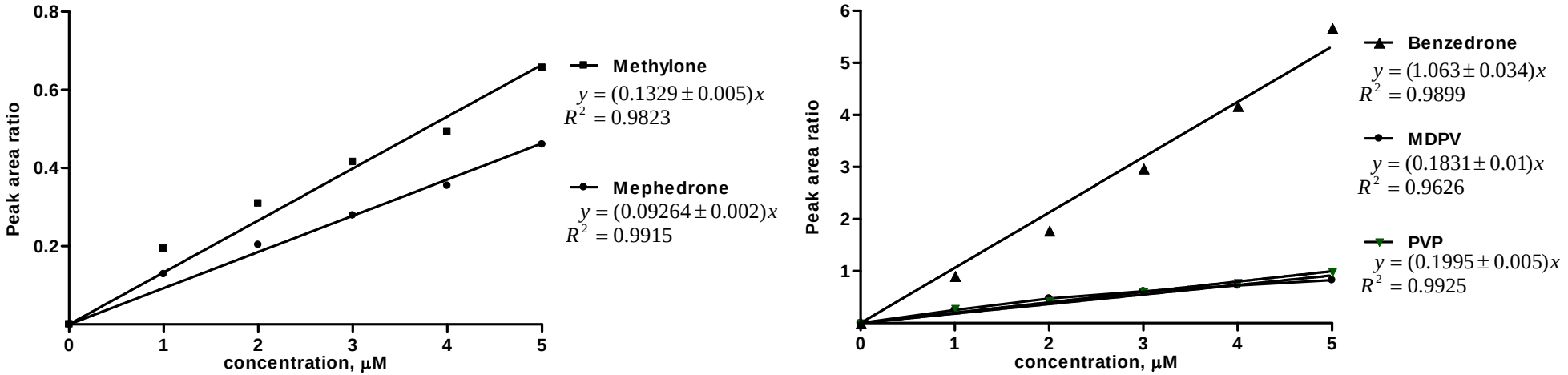
Tab. 4 Comparison of PPB between experimental data (n=6) and data sources available in the literature Indication of 95% CI (95% confidence interval of the mean); RSD (Relative standard deviation); References are given in parenthesis

Drug	Theoretical PPB	Experimental PPB	95% CI	RSD	Bias
Butylone	57% (35)	57%± 8.2	47-66%	14.5%	0%
Cimetidine	15-20% (36)	17%± 3.5	13-21%	20%	0%
Sertraline	99% (35)	97%± 0.7	96-98%	0.8%	2%
DOI	65-79% (35)	61%± 8.7	51-70%	14.5%	4%
THC	97% (22)	97%± 0.3	96-97%	0.4%	0%

5.3 Calibration Model

An unweighted linear regression model was applied to all studied drugs. Figures 3-7 show the best fitting linear regression line and the respective straight-line equation (y=m*x) for all drugs. The coefficient of determination between measured and calculated data values exceeded 0.98 for all drugs both in plasma and filtrate indicating a well fitting of the linear regression lines. Accuracy and precision in the range of 1-5 µM were established based on the fact that all measured concentrations deviated less than 10% from the target concentrations.

1) Calibration curves of **synthetic cathinones** in human plasma



2) Calibration curves of **synthetic cathinones** in ultrafiltrate

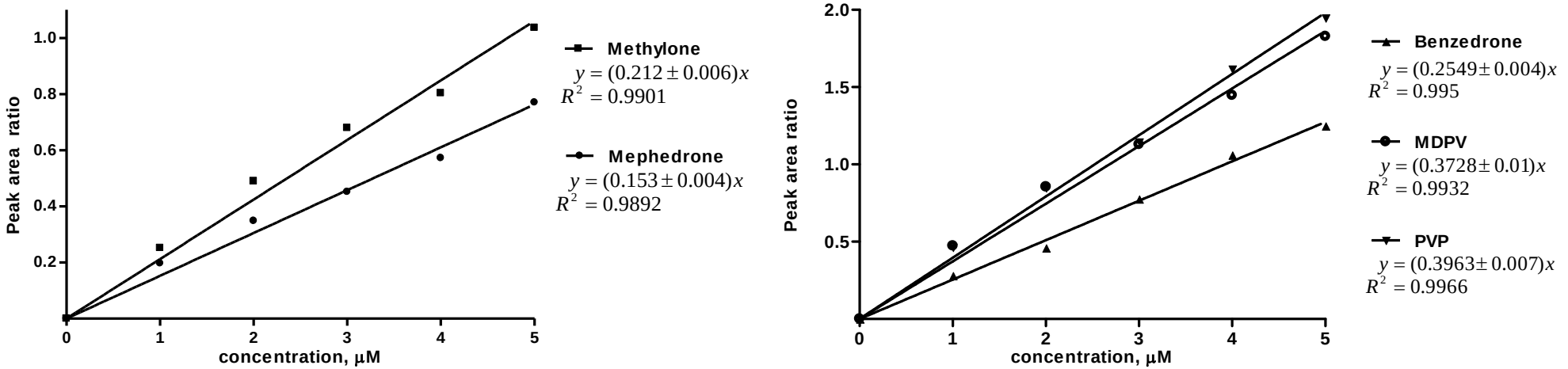


Fig. 3 Calibration curves of synthetic cathinones in the concentration range from 1-5 µM in human plasma (1) and ultrafiltrate (2) *X-axis*: concentration, µM / *Y-axis:* Peak area ratio, NPS/IS

1) Calibration curves of **synthetic cannabinoids** in human plasma

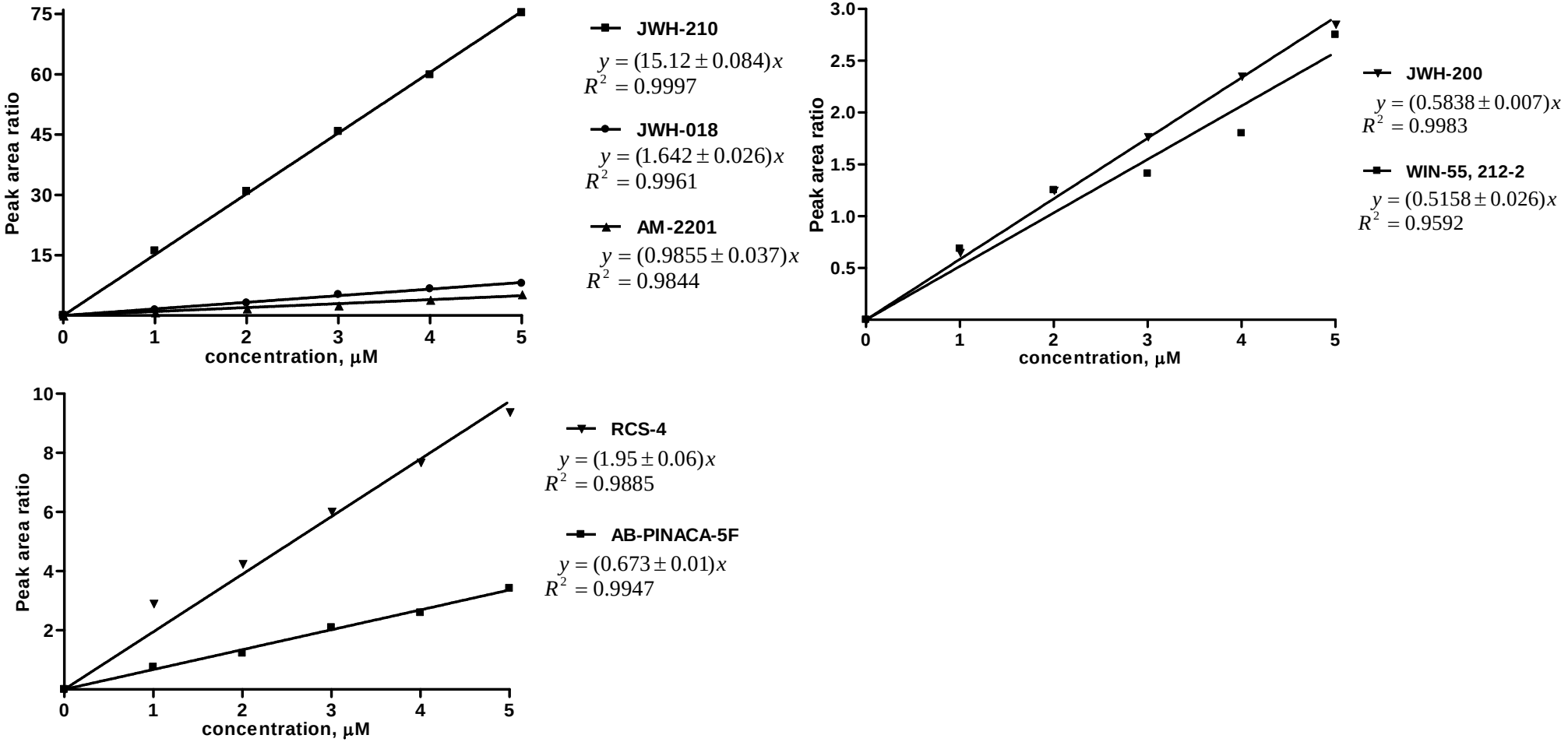


Fig. 4 Calibration curves of synthetic cannabinoids in the concentration range from 1-5 μM in human plasma
X-axis: concentration, μM / *Y-axis:* Peak area ratio, NPS/IS

2) Calibration curves of **synthetic cannabinoids** in ultrafiltrate

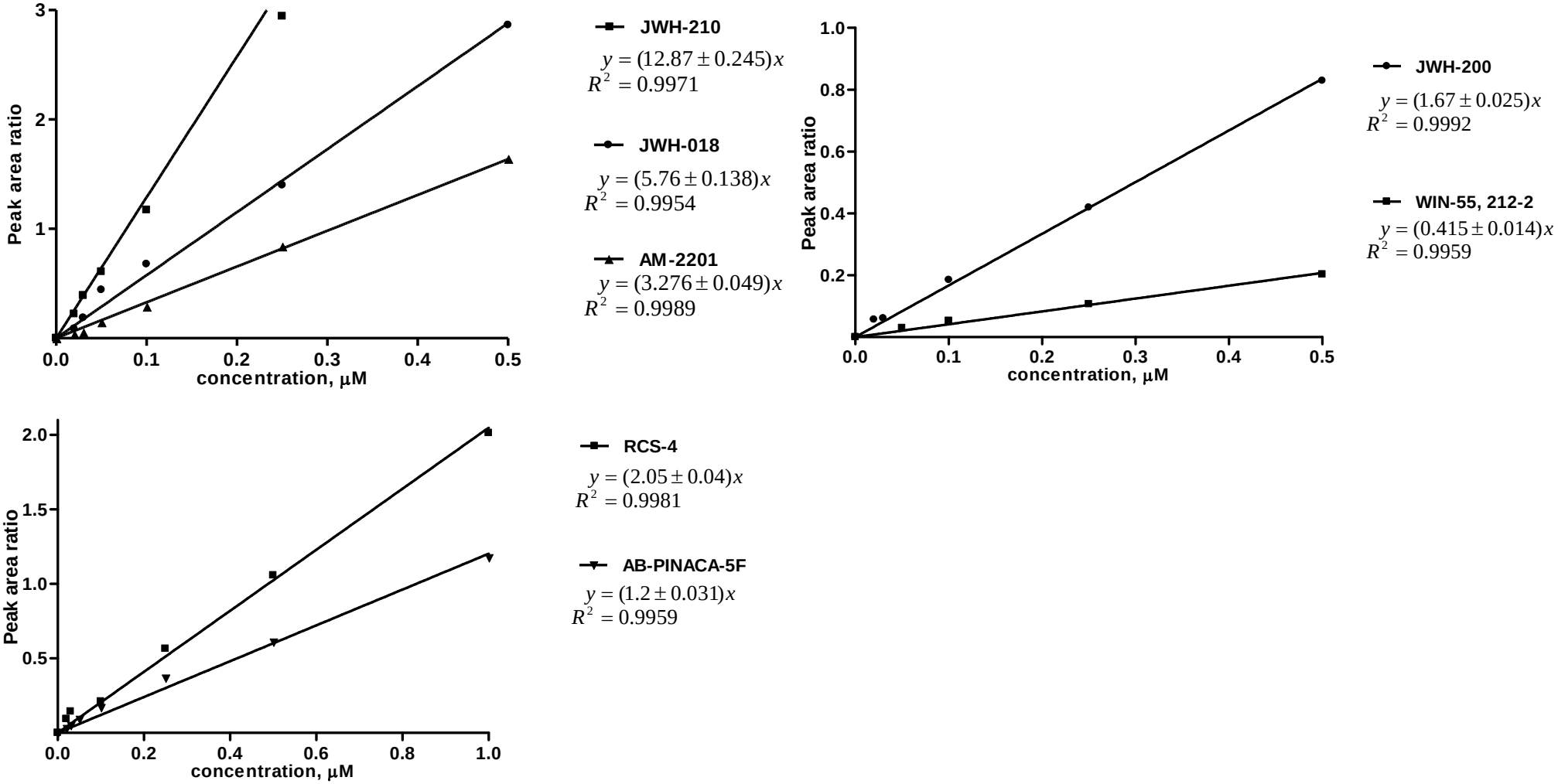
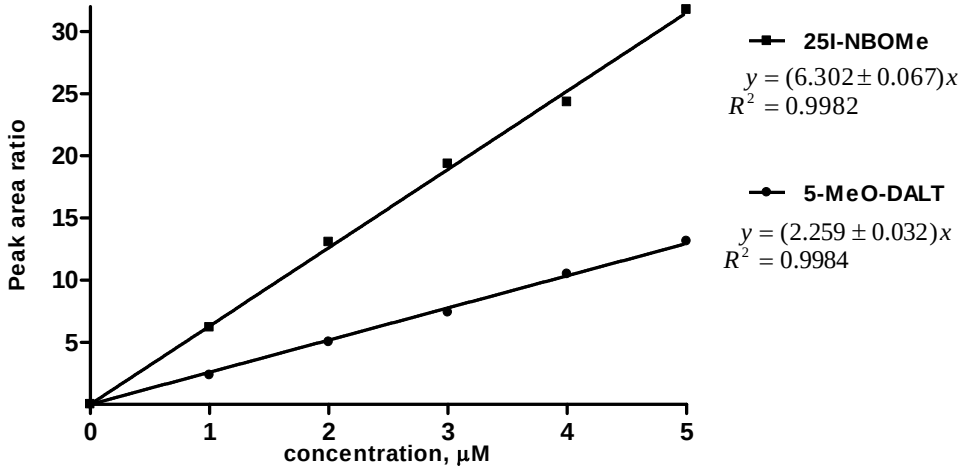
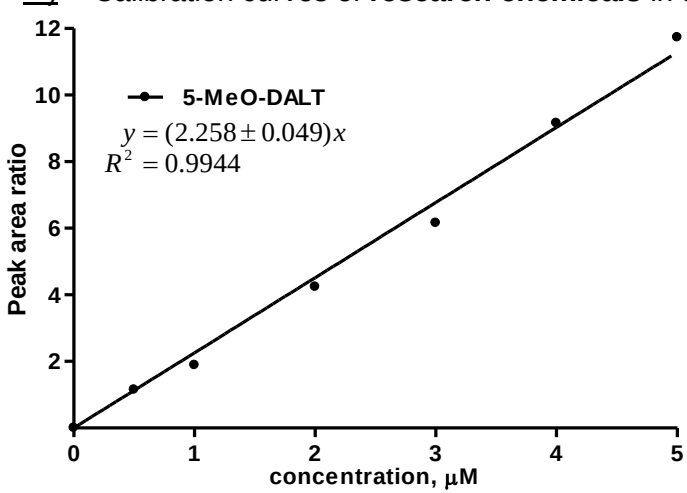


Fig. 5 Calibration curves of synthetic cannabinoids in the concentration range from 1-5 µM in ultrafiltrate
X-axis: concentration, µM / *Y-axis:* Peak area ratio, NPS/IS

1) Calibration curves of **research chemicals** in human plasma



2) Calibration curves of **research chemicals** in ultrafiltrate



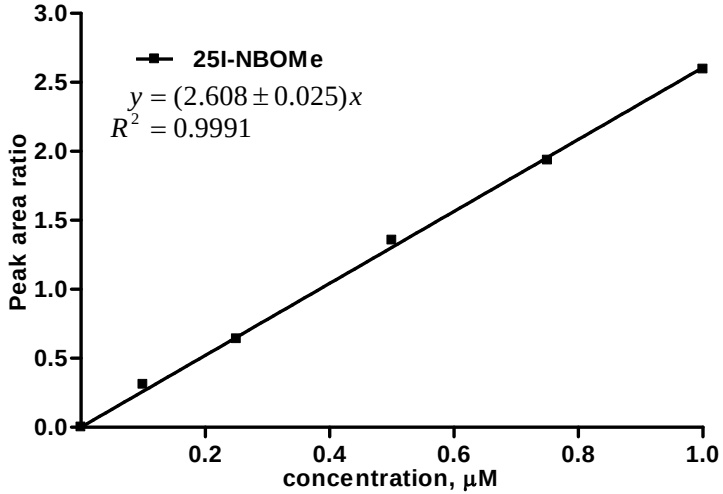
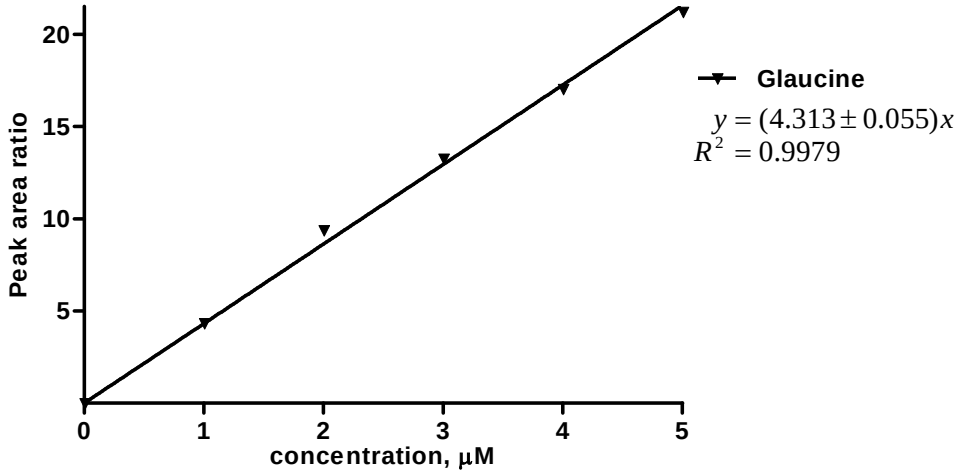


Fig. 6 Calibration curves of research chemicals in the concentration range from 1-5 µM in human plasma (1) and ultrafiltrate (2) *X-axis*: concentration, µM / *Y-axis:* Peak area ratio, NPS/IS

1) Calibration curves of **herbal drugs** in human plasma



2) Calibration curves of **herbal drugs** in ultrafiltrate

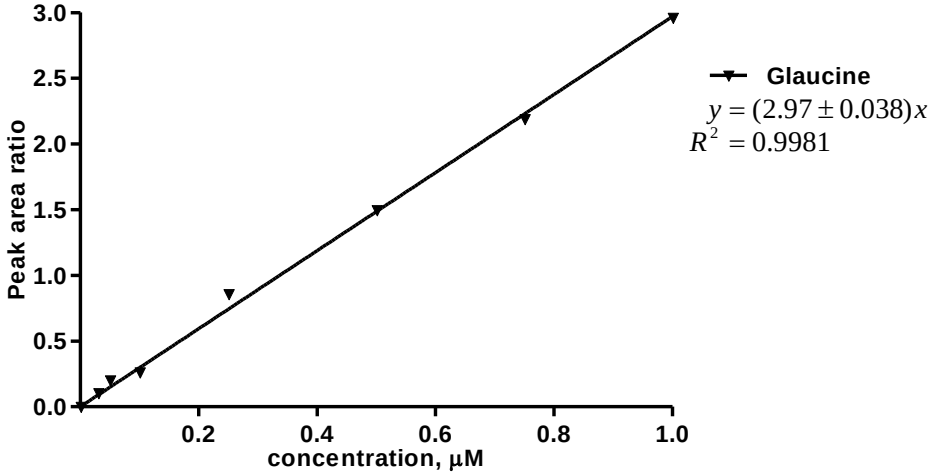
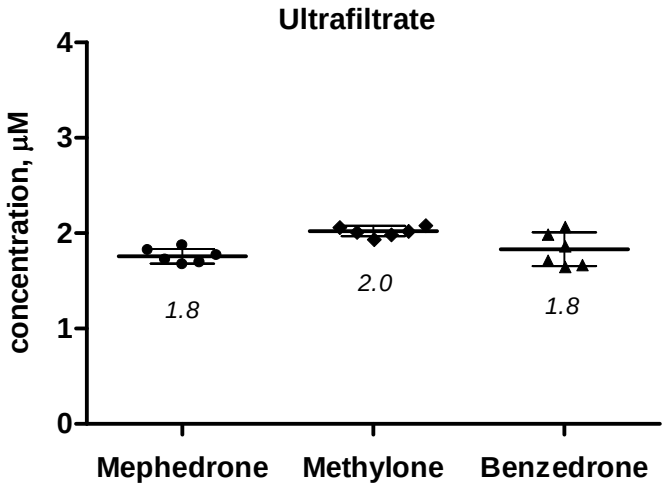


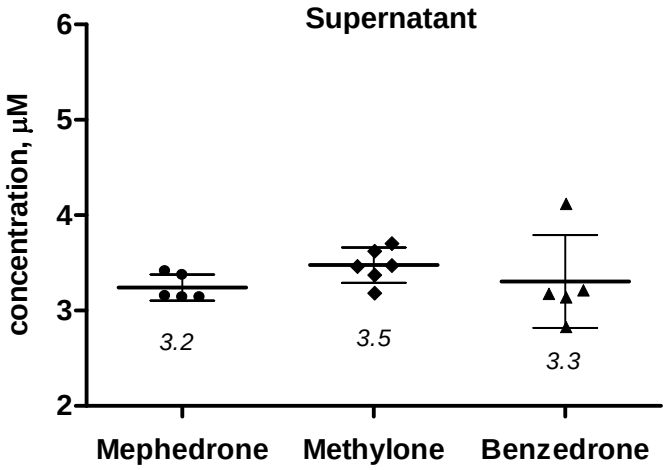
Fig. 7 Calibration curves of herbal drugs in the concentration range from 1-5 μM in human plasma (1) and ultrafiltrate (2) *X-axis*: concentration, μM / *Y-axis:* Peak area ratio, NPS/IS

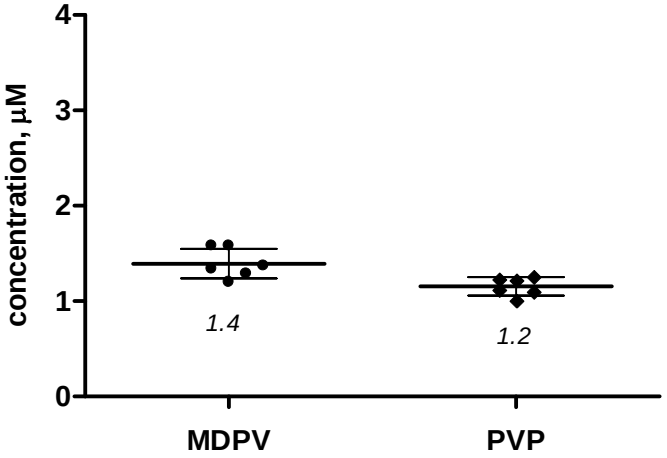
5.4 Plasma Protein Binding Studies

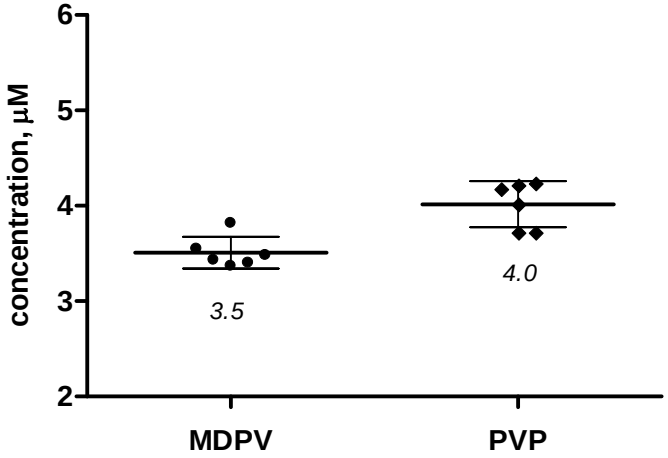
Six replicates of each analyte with an initial concentration of 5 µM in human plasma were measured first as control series and, after ultrafiltration, for determination of PPB in ultrafiltrate and retained volume. Column graphs of the sextuple measured drug concentration in ultrafiltrate and supernatant of all 15 NPS are shown in Figure 8. The respective mean values are outlined in italics in each graphic. The sum of the calculated mean values of each analyte in ultrafiltrate and supernatant did not differ from 5 µM by more than 10%, respectively. Coefficients of variation never exceeded 15% in the concentration range from 1-5 µM and 20% in the lower concentration range from 0.2-1 µM in ultrafiltrate. The measured values in the control series covered the initial concentration of 5 µM within the 95% confidence interval. Corresponding statistical parameters of the control series are given in Tables 5-8. Mean values, standard deviation, and coefficient of variation are reported. All data values were tested on statistical significant outliers performing Grubb`s test with a significance level of α=0.05. Identified outliers were excluded from calculations of PPB. The dispersion of the values highly depended on the applied matrix noticing a greater deviation of the replicates in supernatant compared with the same measurements in ultrafiltrate.

Synthetic cathinones

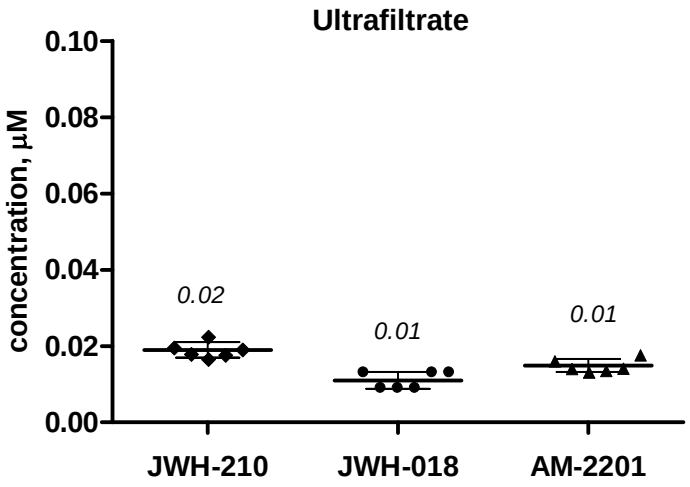


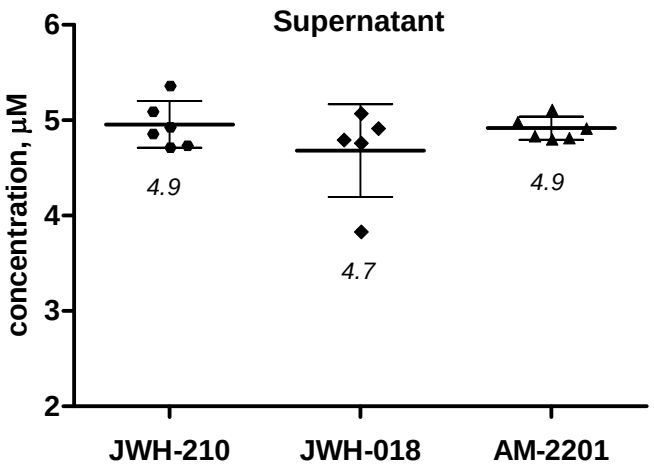


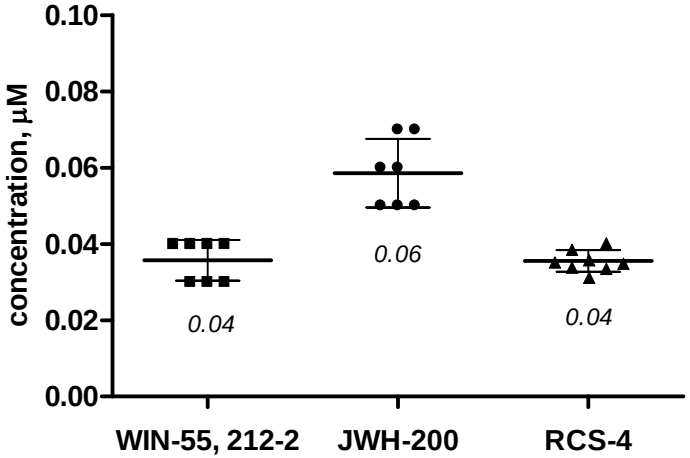


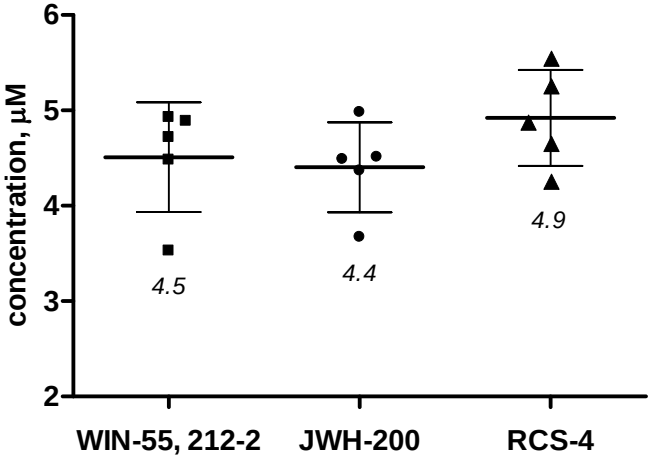


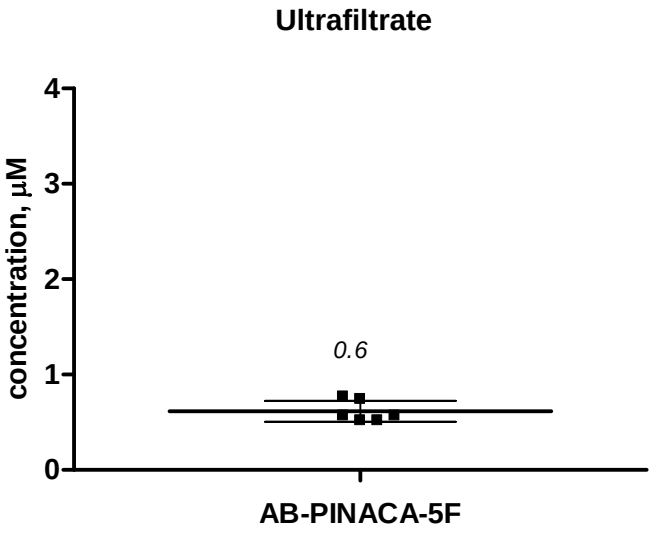
Synthetic cannabinoids

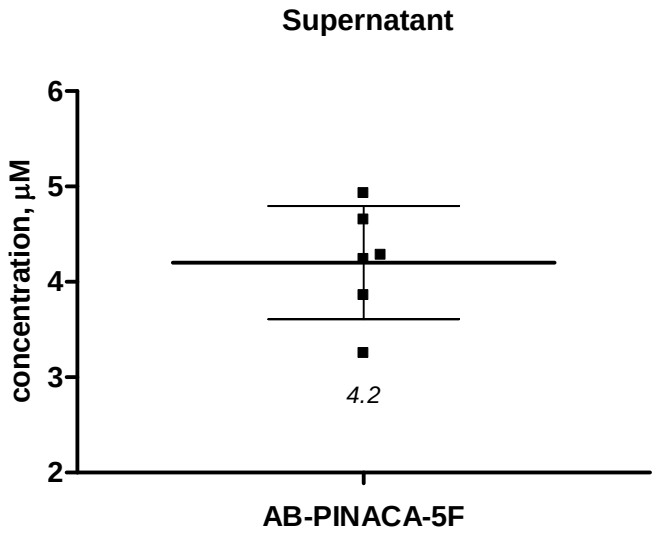


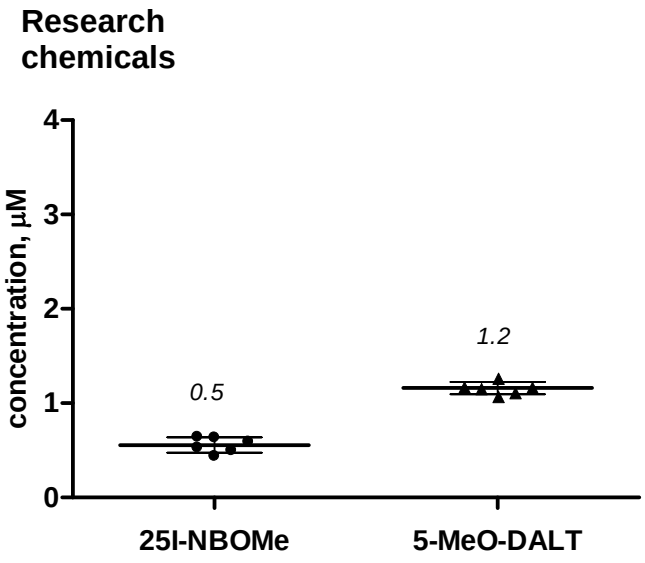


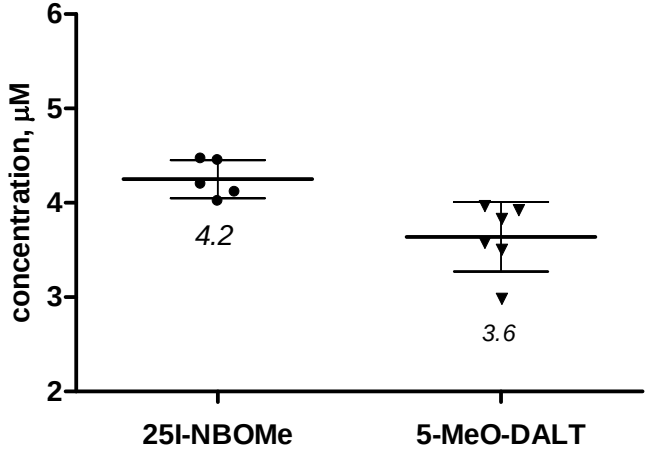





Ultrafiltrate
concentration, µM
4
3
2
1
0
0.6
AB-PINACA-5F


Supernatant
concentration, µM
6
5
4
3
2
4.2
AB-PINACA-5F


Research chemicals
concentration, µM
4
3
2
1
0
0.5
1.2
25I-NBOMe
5-MeO-DALT


concentration, µM
6
5
4
3
2
4.2
3.6
25I-NBOMe
5-MeO-DALT

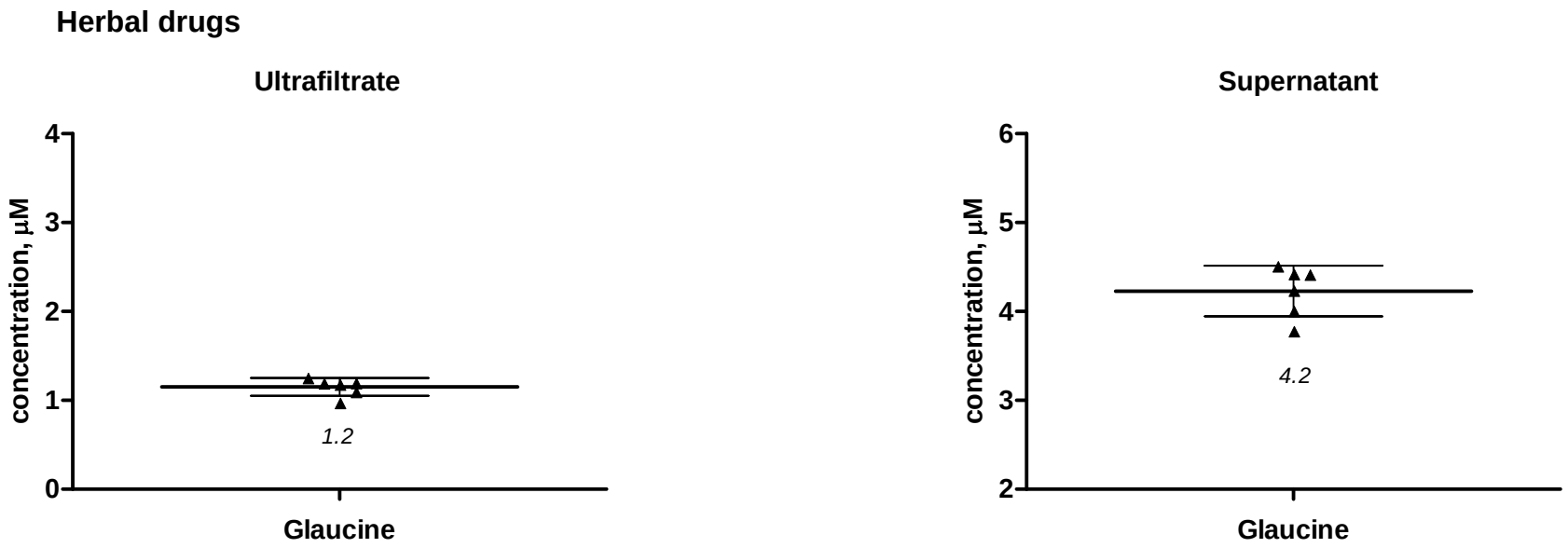


Fig. 8 Column graphs of experimentally determined concentrations of the respective NPS in ultrafiltrate (left side) and supernatant (right side); n=6; Indication of mean in italics

Tab. 5 Synthetic cathinones- Control Series

Statistical parameters of experimental data obtained in human plasma; n=6 Indication of mean, standard deviation (SD), coefficient of variation (CV)

Drug	Mean	SD	CV
Mephedrone	4.8	0.1	2.9 %
Methylone	4.9	0.0	0.8 %
Benzedrone	5.5	0.4	6.7 %
MDPV	4.3	0.2	4.1 %
PVP	4.8	0.7	13 %

Tab. 6 Synthetic cannabinoids- Control Series

Statistical parameters of experimental data obtained in human plasma; n=6 Indication of mean, standard deviation (SD), coefficient of variation (CV)

Drug	Mean	SD	CV
JWH-018	5.3	0.6	12 %
JWH-210	5.3	0.3	6.2 %
AM-2201	5.2	0.3	5.1 %
JWH-200	5.2	0.5	9.3 %
WIN-55,212-2	4.9	0.5	9.5 %
RCS-4	5.0	0.2	4.3 %
AB-PINACA-5F	5.1	0.7	14 %

Tab. 7 Research chemicals- Control Series

Statistical parameters of experimental data obtained in human plasma; n=6 Indication of mean, standard deviation (SD), coefficient of variation (CV)

Drug	Mean	SD	CV
25I-NBOMe	5.6	0.3	4.7 %
5-MeO-DALT	5.1	0.3	5.6 %

Tab. 8 Herbal drugs-Control Series

Statistical parameters of experimental data obtained in human plasma; n=6 Indication of mean, standard deviation (SD), coefficient of variation (CV)

Drug	Mean	SD	CV
Glaucine	5.2	0.4	8.5 %

6 Discussion

6.1 Significance of the Present Study

PPB of drugs is considered to be an important parameter in forensic toxicology as free drug concentrations in body fluids are regulated by protein binding (8). Solely the amount of free drug is detectable in drug-screening tests and moreover crucial for the management of poisoned patients considering the possibility of detoxification therapy (e.g. hemodialysis) only for drugs with a low degree of PPB. Due to the significance of establishing precise PPB values especially for NPS, a sensitive and well practicable technique has been developed.

6.2 Method Development

The method used in the present study turned out to determine reliable data of PPB up to bound fractions of nearly 100%. All investigated NPS showed affinity to plasma proteins with corresponding results in ultrafiltrate and supernatant. It should be noted that the extent of standard deviations was dependent of the used matrix. The standard deviations were always higher in the supernatant. Ultrafiltrate is considered to achieve the most precise measurements. The applied procedure has been found to be suitable for concentrations of 5 µM, which was chosen to cover expected ranges of drug concentrations in body fluids.

There are various methods of isolating the unbound fraction in human plasma samples. In several studies, ultrafiltration is reported as a simple, quick, and safe technique in contrast to the most common alternatives such as equilibrium dialysis and ultracentrifugation (3–5,37). Regenerated cellulose membranes are confirmed to affect experimental studies to a lesser extent by nonspecific adsorption than polyethersulfone and modified polyethersulfone membranes (2,4,5,37). An initial volume of 500 µL of the dissolved drug centrifuged for 30 min was used to obtain a sufficient amount of ultrafiltrate in the shortest possible time. Different ranges of centrifugation time were proven not to impact the resulting unbound drug concentration as reported elsewhere (4). In addition, a different range of gained ultrafiltrate volume has been experimentally verified not to change the equilibrium of unbound and bound drug above the filter (4,37). The pH values were checked to ensure accepted ranges of 7.35-7.45 prior to each experimental procedure. The subsequent analytical step using LC-MS was a sufficient method to identify and quantify the unbound drug. APCI was chosen as soft ionization method based on positive-ion chemical ionization (34). Due to the multidimensional information of the MS presented in signal intensity, mass-to-charge

ratio and chromatographic retention time, all drugs have been determined quickly and clearly. The SIM mode was sufficient to give the most abundant peak signal according to the mass-to-charge ratio (*m/z*) using ions at the parent m/z $(M+H)^+$. Codeine turned out as confident and stable IS without any remarkable drug interferences in the applied chromatographic program.

6.3 Correlation between PPB and Lipophilicity

PPB is commonly associated with physicochemical properties of a drug besides spatial configuration and intermolecular interactions between plasma proteins and drugs (1). This suggests that the extent of PPB is closely linked to the chemical profile of a drug considering little changes in the chemical composition leading to modified extents of PPB. Moreover, it might be assumed that represents of the same drug class exhibit similar extents of PPB. This relationship is further examined in the discussion of PPB results within the respective drug classes below. According to Kratochwil et al., lipophilicity is the most commonly accepted parameter used for prediction of PPB affinity (1). In order to investigate the correlation between PPB and lipophilicity, log P values were used as a valid measure for lipophilicity (27). This parameter outlines the distribution of the unionized drug between the aqueous and organic phase. In Figure 9, the corresponding log P values for each investigated compound have been taken from literature (*PubChem Database*) and were plotted against the experimentally determined PPB values for all 15 NPS (38). Linear correlation was calculated using the Pearson correlation coefficient which was r = 0.8. This result implies a significant correlation between PPB and lipophilicity for the tested compounds (significance level of α=0.05).

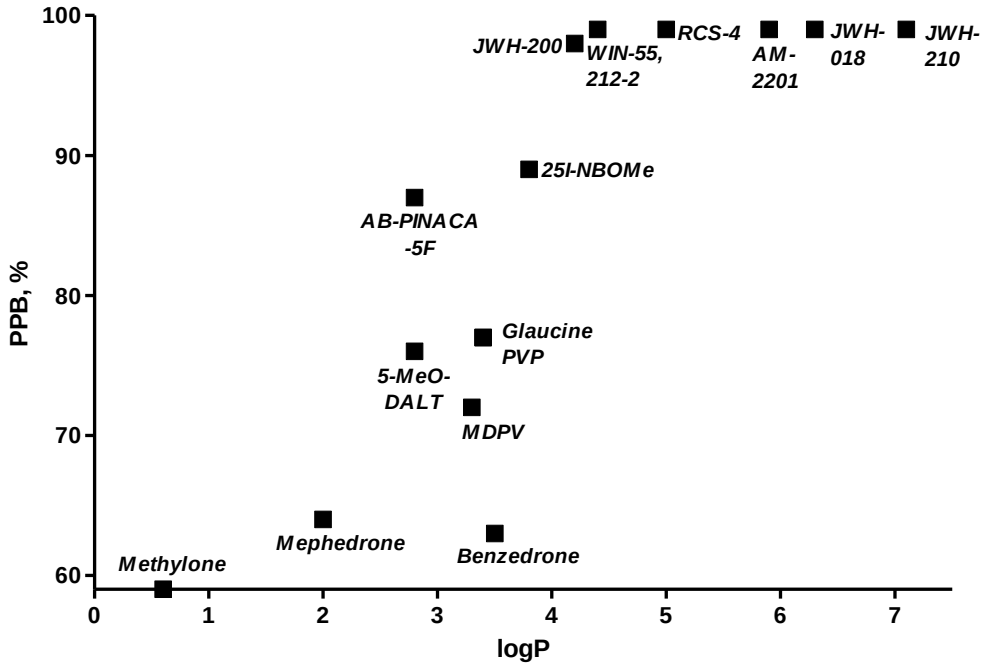


Fig. 9 Correlation between log P (x-axis) and PPB, % (y-axis) for all investigated NPS

6.4 Distribution of PPB-Results within the Classes of NPS

6.4.1 Synthetic Cathinones

[2]

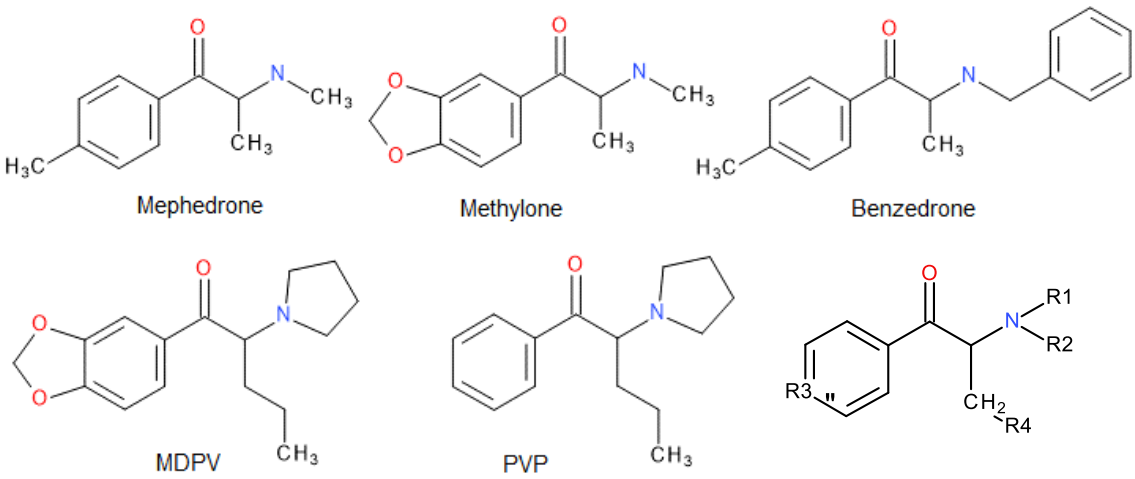


Fig. 10 Chemical structures including a generic structure of the five investigated synthetic cathinones

The five investigated synthetic cathinones show intermediate PPB levels ranging from 59% to 77% (cf. Tab.9). Their chemical profiles differ in substituents of the amino group (R2) and the benzyl ring (R3) as demonstrated in Figure 10. Mephedrone provides a

[2] *BIOVIA Draw 2016* was used to create all following structural formulae

chemical structure closest to the natural cathinone. It is characterized by simple methylation of the primary amino group and a further ring-substitution of a methyl group in para position. By addition of a benzyl ring to the methyl residue of the secondary amino group, a more complex and more lipophilic compound is created. Benzedrone is rather bound to plasma proteins than mephedrone as previously derived. A further subtype of cathinone derivates is represented by methylone containing a methylenedioxy group at R3. Due to the polarizing effect of this functional group, methylone has less lipophilic properties, expressed as well in a lower extent of PPB. MDPV is characterized by the heterocyclic pyrollidine leading to a tertiary nitrogen atom. Its chemical structure includes furthermore the 3,4-methylenedioxy group like methylone. The tertiary amino group leads to a more lipophilic molecule resulting in higher blood–brain barrier permeability than other cathinone derivates (23) and an increasing PPB. PVP provides a chemical composition similar to MDPV only differing in the not substituted benzyl ring. Consequently, PVP shows the highest lipophilicity of all five derivates, resulting furthermore in the strongest binding to plasma proteins.

Tab. 9 Experimental results of PPB in ultrafiltrate (UF) and supernatant (SN) pointed out for the class of synthetic cathinones

Drug	PPB - UF	PPB-SN
Mephedrone	**64%** (61-67)	**62%** (51-71)
Methylone	**59%** (58-61)	**69%** (65-73)
Benzedrone	**63%** (59-67)	**57%** (46-68)
MDPV	**72%** (69-75)	**70%** (66-74)
PVP	**77%** (75-79)	**80%** (75-85)

6.4.2 Synthetic Cannabinoids

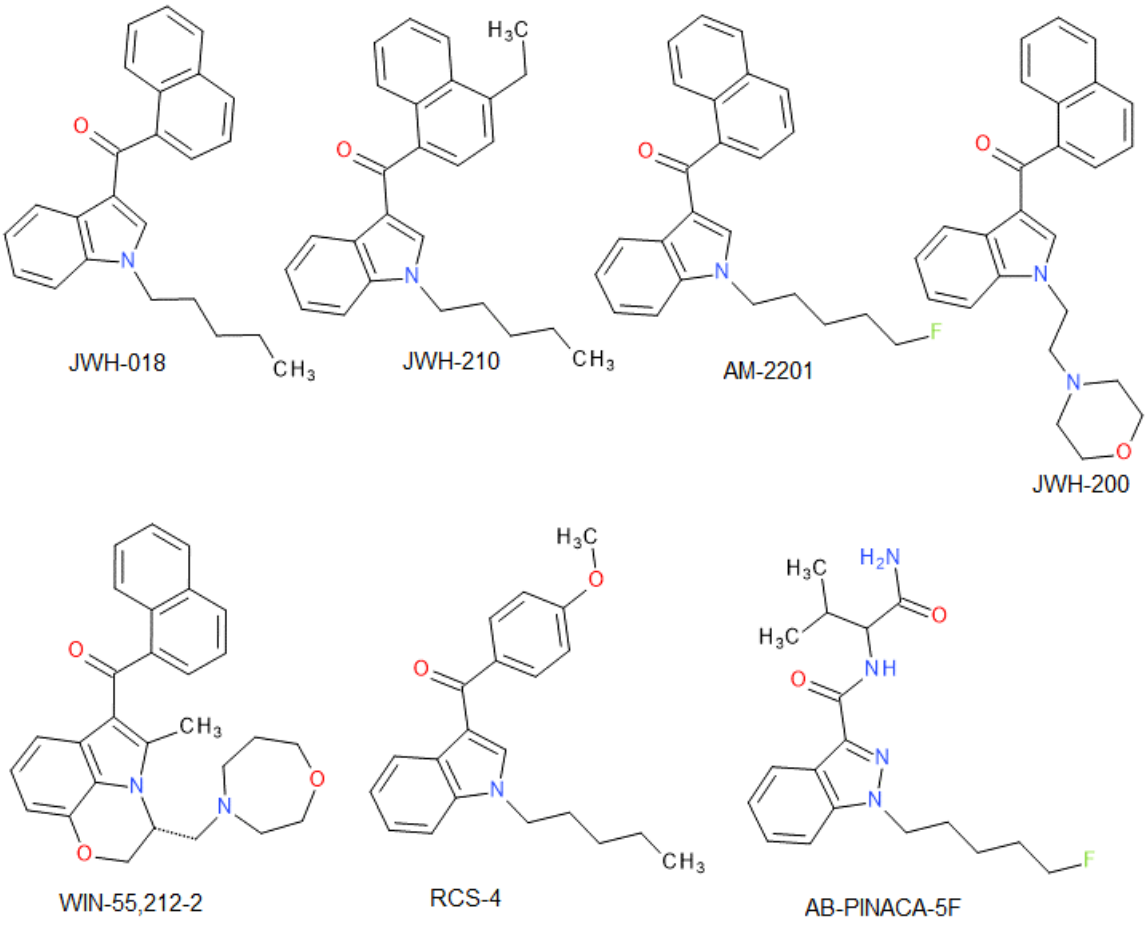


Fig. 11 Chemical structures of the investigated synthetic cannabinoids

Tetrahydrocannabinol (THC) as the principal compound in cannabis is known to be highly bound to plasma proteins based on literature values of 97% (6). Its chemical structure is derived from the class of aromatic terpenoids. Cannabinoids in general are known to be strongly lipophilic and thus present a long elimination half-life compared to other recreational drugs. The class of SC shows PPB levels close to 100% (cf. Tab. 10). As demonstrated in Figure 11, all compounds, except for AB-PINACA-5F, possess a naphthoylindole group as chemical basic. Characteristically, the indole nitrogen is linked to an aryl group resulting in a tertiary nitrogen atom with strongly lipophilic properties. Besides, aminoalkylindoles act as effective CB1 agonists with much higher analgesic potency than other cannabinoids (22,27). JWH-018, JWH-210 and AM-2201 exhibit binding values of 99% in the experimental studies and show a high degree of similarity regarding their chemical pattern. JWH-018 is characterized by a pentyl group linked to the indole nitrogen which is attributable to enhance the psychotropic effects (27) as JWH-018 is 3.4-fold more potent than THC (18). JWH-210 is only differing from

JWH-018 by substitution of an ethyl group at C-4 of the naphthalene providing once again an increase in CB1 receptor affinity (27). AM-2201 is the fluorinated analogue of JWH-018 possessing equal psychotropic properties as well as affinity to plasma proteins. JWH-200 exhibits a more complex functional group linked to the indole nitrogen. First of all, WIN 55,212-2 distinguishes from the compared SC in a methyl residue at the indole group and moreover a modified morpholinyl substitution at the indole nitrogen. The thus gained complex spatial configuration indicates a great fit for human plasma proteins. RCS-4 possesses a methoxyphenyl ring instead of the naphthoyl group in its basic structure resulting in no significant differences in the affinity of PPB. AB-PINACA-5F provides the most significant modifications in the chemical composition with regard to the family of SC. This compound differs from all other investigated SC in the indazole nucleus linked to an aliphatic fluoropentyl chain and a carboxamide derivate. It is the fluorinated analogue of AB-PINACA. In particular, the carboxamide groups leads to a polarized and hydrophilic molecule. These structural differences are mainly apparent in the significantly lower PPB compared with the previous values.

Tab. 10 Experimental results of PPB in ultrafiltrate (UF) and supernatant (SN) pointed out for the class of synthetic cannabinoids

Drug	PPB - UF	PPB-SN
JWH-018	**99%** (99-100)	**100%** (89-100)
JWH-210	**99%** (99-100)	**99%** (94-100)
AM-2201	**99%** (99-100)	**98%** (96-100)
JWH-200	**98%** (98- 99)	**88%** (78- 97)
WIN-55,212-2	**99%** (99-100)	**90%** (78-100)
RCS-4	**99%** (99-100)	**98%** (88-100)
AB-PINACA-5F	**87%** (85- 90)	**84%** (72-96)

6.4.3 Research Chemicals

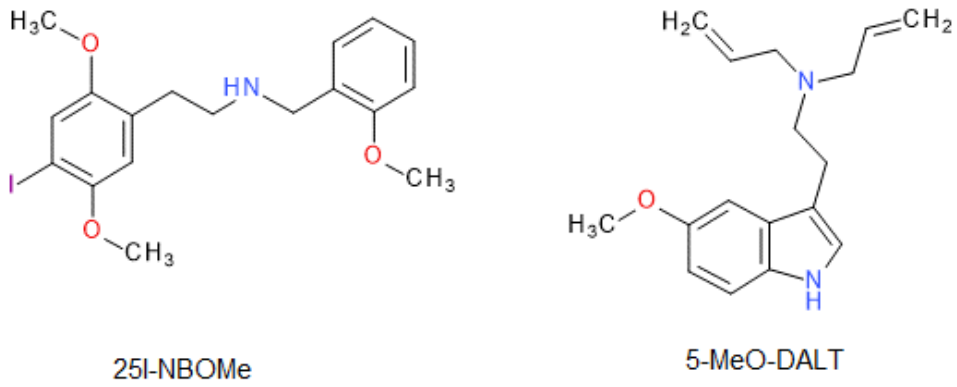


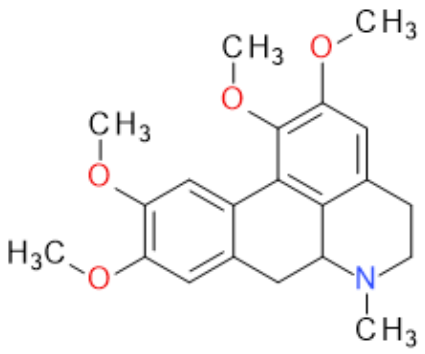
Fig. 12 Chemical structures of the investigated research chemicals

The two investigated research chemicals originate from two different chemical families. 25I-NBOMe belongs to the chemical group of 2,-5-dimethoxyphenethylamines, which is characterized by addition of methoxy- groups at the 2- and 5-positions of the aromatic ring (cf. Fig. 12). This structural modification, as well as the iodation at position 4 of the ring, increases the 5-HT_{2A} receptor affinity compared with classic hallucinogens. Furthermore, the chemical structure of 25I-NBOMe provides a secondary nitrogen atom substituted with a methoxy phenylring. Due to the aromatic ring systems and the oxygen substituents with electron donor effects, the molecule acts as a chemically stable and lipohilic compound with a high PPB (cf. Tab. 11). 5-MeO-DALT possesses an indole nucleus as basic structure linked to an ethylamine group with two attached propionyl side chains. The tertiary amino group creates a molecule with high lipophilic properties. Furthermore, by introducing an alkylated hydroxyl group at position 5 of the aromatic ring, increased permeability of the blood-brain barrier and consequently great potency is achieved (12). 5-MeO-DALT provides a high binding affinity to plasma proteins in the experimental series.

Tab. 11 Experimental results of PPB in ultrafiltrate (UF) and supernatant (SN) pointed out for the class of research chemicals

Drug	PPB - UF	PPB-SN
25I-NBOMe	**89%** (87-91)	**85%** (80-89)
5-MeO-DALT	**76%** (75-78)	**71%** (67-80)

6.4.4 Herbal Drugs



Glaucine

Fig. 13 Chemical structures of the investigated herbal drugs

Glaucine represents the chemical class of isoquinoline alkaloids containing a benzopyridine as basic structure. This functional group is characterized by four methoxy units connected to two merged benzene rings. The tertiary amine in the piperidine ring leads to a more lipophilic compound. The aromatic system is stabilized by the methoxy groups referred to above, acting as free electron pair donors. Due to lipophilic basic structure along with the tertiary amine, the experimental findings are well corresponding to the structural composition as demonstrated in Table 12. Furthermore, the tertiary amino group linked to an aromatic system was accepted as essential structural characteristic concerning P-gp interactions (28). Fulfilling this criterion, JWH-200, WIN 55,212-2 and glaucine were investigated and identified as inhibitors of P-gp causing possible toxic effects by increasing the extent of oral absorption of P-gp substrates (28).

Tab. 12 Experimental results of PPB in ultrafiltrate (UF) and supernatant (SN) pointed out for the class of herbal drugs

Drug	PPB - UF	PPB-SN
Glaucine	**76.9%** (74.9-78.9)	**84.6%** (78.9-90.3)

Summarizing, all NPS display a trend towards high PPB levels. In the separate classes, the individual compounds exhibit comparable extents of PPB. It is therefore obvious that molecules with closely related chemical structures show a very similar tendency of PPB. This is evidenced considering little changes in the basic structure of a family class leading to modified values of PPB as underpinned, inter alia, with the experimental results of AB-PINACA-5F.

7 Conclusion

PPB was successfully determined for 15 NPS representing synthetic cathinones, synthetic cannabinoids, research chemicals and herbal drugs. Values reaching from 67% to >99% for the respective drugs were determined indicating a strong tendency of high PPB for SC. In addition, the developed method turned out as a fast and simple technique practicable in most common forensic and clinical laboratories. Due to the importance of the bound ratio of drugs in human blood considering the direct impact on important pharmacological parameters, this method could serve as valuable research method in prospective screening procedures of novel psychoactive drugs.

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